Supplementary information

Hexameric assembly of the proteasomal ATPases is templated through their C-termini

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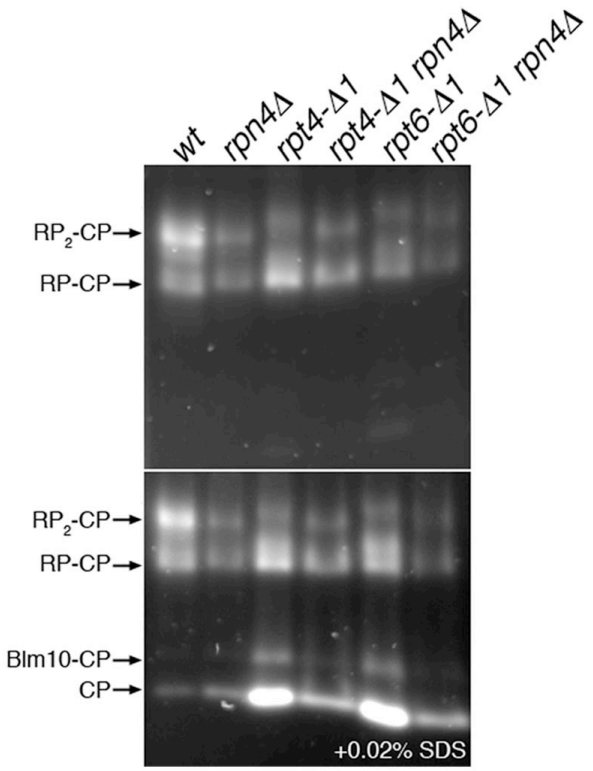
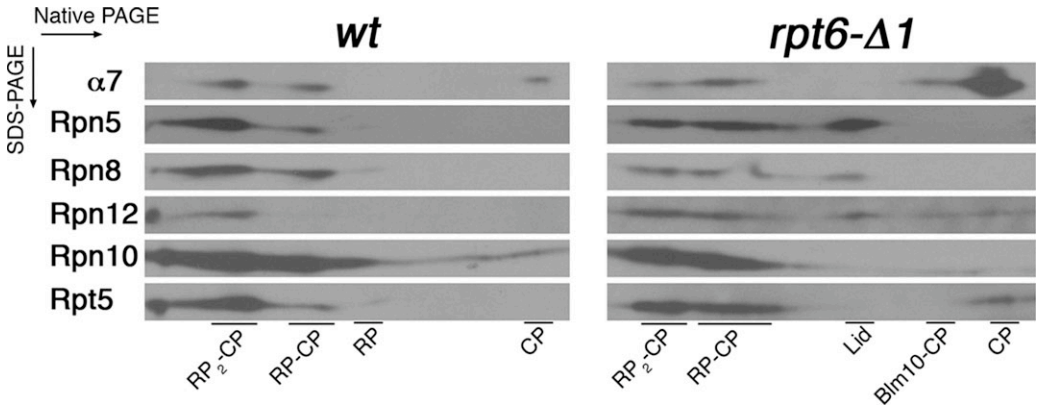
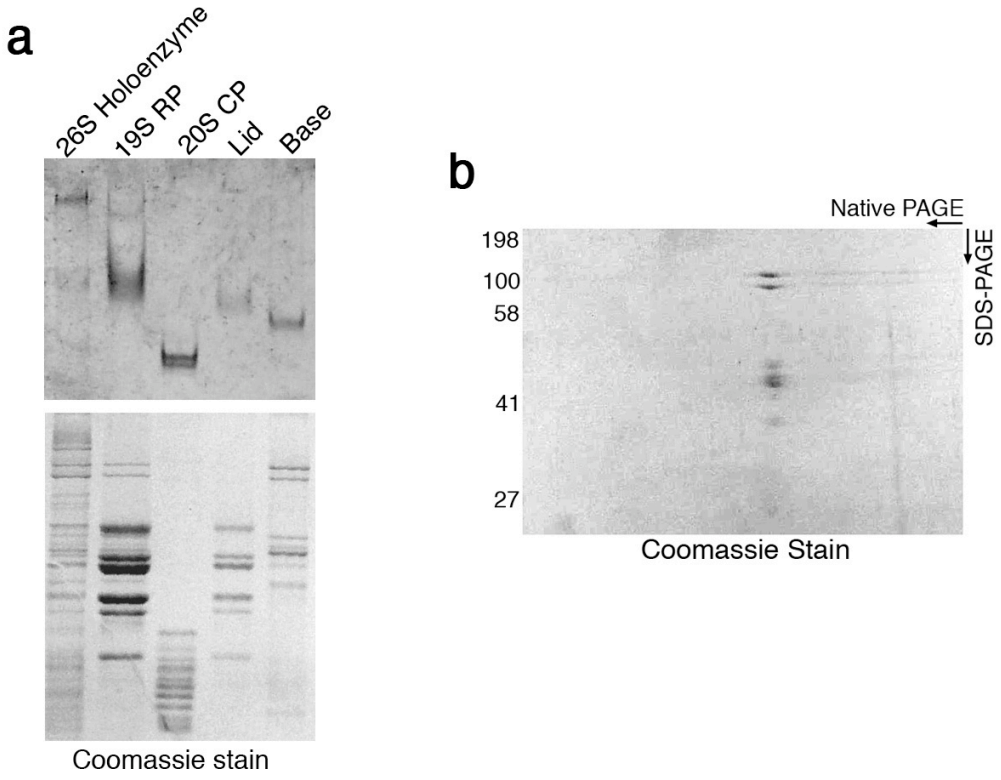


Supplementary Figure 1. Deletion of *RPN4* results in a reduction in the level of free CP in *rpt4-Δ1* and *rpt6-Δ1* mutants. These data complement Figure 1c. Native PAGE (3.5%) and two consecutive LLVY-AMC assays with whole cell extracts (85 μg). Immediately after the first LLVY-AMC assay (top panel), the second assay was conducted in the presence of 0.02% SDS (bottom panel).



Supplementary Figure 2. The *rpt6-Δ1* mutant produces free lid species containing multiple subunits of the lid. These data complement Figure 1f. 2-D native/SDS-PAGE analysis with whole cell extracts (85 μg). Following the native PAGE (3.5%), SDS-PAGE (4-12%) and immunoblotting were performed to detect free lid species using antibodies to lid subunits (Rpn5, Rpn8, and Rpn12). Base subunits (Rpn10 and Rpt5) and a CP subunit (α7) were used as controls.

Although a small fraction of Rpt5 comigrates with CP in the *rpt6-Δ1* sample, this is not taken to indicate complex formation, based on other data.



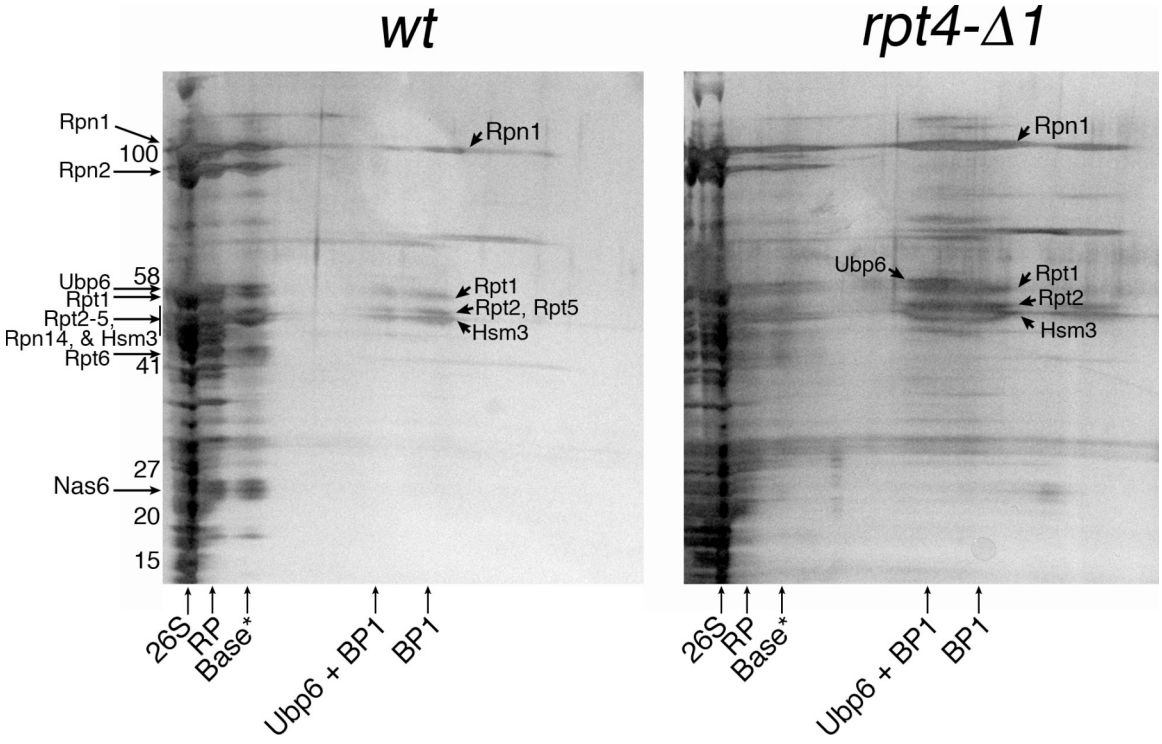
Supplementary Figure 3. Analysis of purified base by native PAGE.

The base, in its free form, has not been observed to migrate on native gels, which has hindered analysis of base assembly. We found that lysing cells in liquid nitrogen resulted in purified base samples that migrated into native gels (see Supplementary Methods).

a, Affinity-purified proteasomes and proteasomal subcomplexes were subjected to 3.5% native PAGE (top panel), or 12.5% SDS-PAGE (bottom panel) and stained with Coomassie. Affinity purifications were conducted using a TeV-ProA tag appended to Rpn11 (26S holoenzymes, 19S RP, lid), Pre1 (CP), or Rpt1 (base). The ProA tag is N-terminal to Rpt1, and C-terminal to Rpn11 or Pre1.

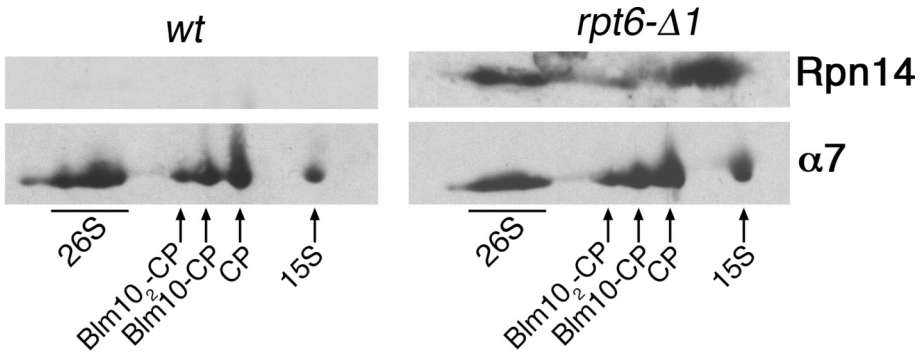
b, 2-D native/SDS-PAGE (3.5% and 12.5%, respectively) of the purified base. Upon the completion of first dimension native PAGE with affinity-purified base as in (**a**), the entire

lane containing the base was excised and subjected to the second dimension SDS-PAGE (12.5%). The gel was stained with Coomassie. This result verified that the observed species on native gels was bona fide base.



Supplementary Figure 4. 2-D native/SDS-PAGE analysis and silver staining of samples containing BP1.

Affinity-purification using ProA-TeV-Rpt1 was conducted from the indicated strains. 2-D native/SDS-PAGE (5% and 4-12%, respectively) as in Figure 2a was then conducted, followed by silver stain. Labels on the left indicate the spots derived from base*. The heavy Ubp6 spot seen at the position of BP1+Ubp6 (in the *rpt4-Δ1* sample), and the overall intensity of protein components of the BP1+Ubp6 complex, are reduced when 150 mM NaCl wash was conducted instead of 50 mM NaCl wash during the purification (data not shown), consistent with salt-dependent association of Ubp6[1] with the proteasome.



Supplementary Figure 5. Association of Rpn14 with proteasomes from *rpt6-Δ1* mutants.

These results complement the Figure 4a.

Proteasomes purified via Pre1-TeV-ProA was resolved by 2-D native/SDS-PAGE (3.5% and 12.5%, respectively), and subjected to immunoblotting for Rpn14. Proteasome holoenzyme species and other CP species were visualized using an antibody against α7 (a CP subunit).

Note that a fast-migrating form of Rpn14 is seen only in the Rpt6-Δ1 sample. This is possibly Rpn14 that has dissociated from the 26S proteasome after the purification step. The Rpn14 streak to the right of the CP species might reflect dissociation of Rpn14 during electrophoresis.

Supplementary Methods

Yeast strains and media. All yeast manipulations were conducted according to standard protocols[2]. Strains used in this study are listed in Supplementary Table 2. C-terminal single deletions in each of the six *RPT* genes were made in their endogenous chromosomal loci by integrative recombination as essentially described in a previous study[3]. Strains were grown in YPD medium to OD_{600} 0.8-1.2 for the analysis of BP1, or to OD_{600} 2.0-4.0 for the analysis of mature proteasomes.

Native PAGE, in-gel peptidase assay, 2-D native/SDS-PAGE and immunoblot. Proteasome holoenzymes were analyzed by 3.5% native PAGE and in-gel peptidase assay as described previously[4]. BP1 and base complexes were resolved by 5% discontinuous native PAGE using conditions described in a previous study[5]. For immunoblotting, native gels were incubated in 1X SDS-PAGE running buffer[6] (or transfer buffer[6] containing 0.1% SDS) for 15 min and transferred to PVDF at 80mA overnight at 4°C. The procedure for 2-D native/SDS-PAGE is described in detail in a previous study[6]. Rpn5, Rpn8, Rpn12, eIF5A antibodies were previously described[7]. Antibodies against Rpt5 and α7 were from Biomol and were used at 1:2000. Rpt1 antibody was used as previously described[1]. RP-chaperone antibodies are described in the accompanying study[8].

Purification of proteasomal subcomplexes. 26S proteasome holoenzyme, 19S RP, 20S CP, and lid were affinity-purified using a ProA tag in the presence of 1 mM ATP and 5 mM $MgCl_2$ following previously described procedures[4]. For the affinity-purification of the base, cells were lysed under liquid nitrogen as described in the Methods Summary and were subjected to a

previously described procedure[4]. Purified base resulting from liquid nitrogen lysis migrated on native gels.

The endogenous base (base*) and the BP1 complexes were affinity-purified as described in the Methods Summary. During purification, the beads were washed with 50 mM NaCl. Increasing the concentration of salt in the wash buffer to 150 mM NaCl released Ubp6 from the Ubp6 + BP1 complex, but did not disrupt the integrity of the base* or BP1 complexes (data not shown).

Mass spectrometry of BP1 subunits. BP1 subunit bands were Coomassie-stained following 2-D native/SDS-PAGE. Each subunit band was excised and digested in-gel with trypsin. The resulting peptides were separated by nanoscale reversed phase liquid chromatography coupled to a tandem mass spectrometer (LTQ and LTQ XL; Thermo Electron). Each MS scan was followed by collecting ten MS/MS spectra from the most abundant ions[9]. MS/MS spectra were searched against a yeast database using the Sequest algorithm[10].

Cell lysis. French press lysis was used for experiments shown in Figures 1a and 1f, and Supplementary Figure 2. Liquid nitrogen lysis as described in Methods Summary was used for experiments shown in Figures 2, 3, 4a, 4b and 4c, and Supplementary Figures 1, 3, 4, and 5.

Pulse-chase analysis of BP1. Strains were grown in 1 L of SD medium to OD_{600} 0.6. Cells were then harvested and resuspended in 1 L of SD-methionine medium for 1 hr. Cells were pelleted and divided into two 25 ml–samples in SD-methionine medium. Each sample was pulsed with 3.5 mCi of S^{35}-methionine using EasyTag EXPRESS S^{35} protein labeling mix (Perkin Elmer) for

5 min (*PROA-TEV-RPT1 rpt4-Δ1*) or 4 min (*PROA-TEV-RPT1*), and labeled samples were harvested by centrifugation at 3000 x g for 2 min at 4°C. For the pulsed sample, the labeled cells were drop-frozen in liquid nitrogen immediately. For chased sample, the labeled cells were resuspended in 25 ml of SD medium with excess methionine freshly added to 0.5 mg/ml final concentration and further incubated at 30°C for 30 min. At the end of the chase, cells were harvested at 3000 x g for 2 min and drop-frozen in liquid nitrogen. Frozen yeast cells were then ground under liquid nitrogen with a pestle in a mortar nestled in dry ice. BP1 was immunoprecipitated via the ProA tag and eluted with AcTEV protease as described in the Methods Summary. The entire eluate was subjected to 2-D native/SDS-PAGE (5% and 4-12%, respectively). Gels were dried at 80°C for 1 hr and exposed to x-ray film at -80°C with intensifier screen for 4 days (*PROA-TEV-RPT1 rpt4-Δ1*) to 2 months (*PROA-TEV-RPT1*).

Supplementary Table 1. C-terminal sequences of gating Rpts and templating Rpts

Gating Rpts	
Rpt2	VMKNKVEENLEGLYL
Rpt3	QVKTDNTVDKFDFYK
Rpt5	SEVQARKSKSVSFYA
Putative Gating Rpt	
Rpt1	GYKKFSSTSRYMQYN
Templating Rpts	
Rpt4	AEVKKLEGTIEYQKL
Rpt6	NKNQETAISVAKLFK

Note: Hb-Y-X motif in gating Rpts are highlighted in red. It should be noted that, although the presence of a conserved penultimate tyrosine in Rpt1 suggests that Rpt1 may contribute to CP gating, this has not been shown experimentally. It is also unclear whether the divergence from apparent consensus at the antepenultimate (Q) residue in Rpt1 is functionally significant.

Supplementary Table 2. Yeast strains used in this study

Strain	Genotype	Figure
SUB62[11]	*MAT***a** *lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1*	1, 2c, 2d and 4d
SP4	*MAT***a** *lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt1::rpt1-Δ1-kanMX6*	1a, 1b, 1d, and 1f
SP66	*MAT***a** *lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt2::rpt2-Δ1-kanMX6*	1a, 1b, 1d, and 1f

SP11	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt3::rpt3-Δ1-kanMX6*	1a, 1b, 1d, and 1f
SP602	*MAT**a** lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt4::rpt4-Δ1-natMX4*	1 and 2c
SP546-1	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt5::rpt5-Δ1-natMX4*	1a, 1b, 1d, and 1f
SP314	*MAT**a** lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt6::rpt6-Δ1-natMX4*	1a, 1b, 1d, 1e, 1f, 2c and 4d
SP600	*MAT**a** lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpn4:: kanMX6*	1c, 1e, and 2c
SP603	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt4::rpt4-Δ1-natMX4 rpn4::kanMX6*	1c, 1e and 2c
SP856	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt6::rpt6-Δ1-natMX4 rpn4::kanMX6*	1e and 2c
SY36[4]	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt1::HIS3 pEL36 (TRP1)*	2a, 2b, 2e, and 3
SP712-1	*MAT**a** lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt4::rpt4-Δ1-natMX4 rpt1::HIS3 pEL36 (TRP1)*	2a, 2b, 2e, and 3
SP1086	*MAT**a** lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 hsm3::kanMX6 nas6::TRP1 rpn14::hphMX4*	2d
SP846	*MAT**a** lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt4::rpt4-Δ1-natMX4 hsm3::kanMX6 rpt1::HIS3 pEL36 (TRP1)*	2e
SP876	*MAT**a** lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt4::rpt4-Δ1-natMX4 rpn14::hphMX4 rpt1::HIS3 pEL36 (TRP1)*	2e
SP1108	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt4::rpt4-Δ1-natMX4 nas6::TRP1 rpt1::HIS3 pEL36 (TRP1)*	2e
SP844	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1*	2e

	hsm3::kanMX6 rpt1::HIS3 pEL36 (TRP1)	
SP852-1	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpn14::hphMX4 rpt1::HIS3 pEL36 (TRP1)*	2e
SP1106	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 nas6::TRP1 rpt1::HIS3 pEL36 (TRP1)*	2e
sDL135[4]	*MAT**a** lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 pre1::PRE1-TEVProA (HIS3)*	2b, 4a, 4b, and 4c
SP797	*MAT**a** lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 pre1::PRE1-TEVProA (HIS3) rpt6::rpt6-Δ1- natMX4*	4a
SP419	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 pre1::PRE1-TEVProA (HIS3) rpt3::rpt3-Δ1- kanMX6*	4b
SP985	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 pre1::PRE1-TEVProA (HIS3) nas6::TRP1*	4b
SP1157A	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 pre1::PRE1-TEVProA (HIS3) rpt6::rpt6-SES-hphMX4*	4c
SP1159A	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 pre1::PRE1-TEVProA (HIS3) rpt6::rpt6-S-hphMX4*	4c
SP1156A	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt6::rpt6-SES-hphMX4*	4d
SP1158A	*MATα lys2-801 leu2-3, 2-112 ura3-52 his3-Δ200 trp1-1 rpt6::rpt6-S-hphMX4*	4d

All strains used are congenic to SUB62.

Supplementary references

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